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TITLE: Role of the XIAP/AIF Axis in the Development and Progression of Prostate Cancer

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14. ABSTRACT: Over the project period, significant progress was made towards completing the tasks outlined in the original Statement of Work. The physical association between XIAP and AIF was confirmed in living cells, and was determined to be highly dependant upon the ubiquitination status of AIF. This interaction could be disrupted by Smac/DIABLO, in a manner that correlated with AIF ubiquitination. AIF was shown to be a substrate for XIAP-mediated ubiquitination, which did not result in AIF degradation. The caspase inhibitory properties of XIAP were found to be insensitive to AIF expression and dispensable for AIF binding. AIF overexpression resulted in significant increases in cellular reactive oxygen species levels that were not attenuated by co-expression of XIAP. It was shown that the XIAP antagonist and serine protease Omi/HtrA2 was capable of cleaving AIF, yet the loss of Omi/HtrA2 did not affect the cytoplasmic release of AIF during apoptosis. Finally, AIF was found to associate with the XIAP homologues cIAP-1 and cIAP-2, suggesting that AIF may be a general purpose IAP binding protein. Taken together, these findings not only confirm the physiological relevance of the association between XIAP and AIF, but also establish a functional link between XIAP and AIF, and form the basis for understanding how these two molecules contribute to the development and progression of prostate cancer.

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Introduction

As the second most common form of cancer diagnosed among men in the western world, prostate cancer represents a significant healthcare threat, in part due to the lack of reliable biomarkers to predict disease stage and behavior, as well as a lack of understanding at the molecular level as to the events giving rise to prostate tumorigenesis and metastasis. As part of my original research proposal, I described the identification of two well known regulators of programmed cell death, X-linked inhibitor of apoptosis (XIAP) and apoptosis inducing factor (AIF), as genes that are upregulated in a number of prostate tumors, and further presented data confirming a physical association between the XIAP and AIF proteins. Since these two genes represent both pro-survival (XIAP) and pro-death (AIF) regulators of the cell death cascade, I proposed to investigate the functional relevance of the physical association between XIAP and AIF, in terms of the described functions of both proteins, as well as their potential role in the pathogenesis of prostate cancer. This report summarizes the progress made during the project period towards completing the research proposal goals, as well as additional findings relevant to, but outside of, the original statement of work.

Body

I have made the following progress towards completing the tasks outlined in my original Statement of Work, as summarized below:

AIM 1. To determine the effects of AIF on XIAP function.

a) **Characterization of the XIAP/AIF interaction under normal and apoptotic conditions.** The interaction of XIAP and AIF was confirmed under normal cellular conditions through live cell imaging using Bi-molecular fluorescence complementation. I showed that the Δ102 form of AIF (see below) strongly interacts with XIAP under non-apoptotic conditions (Figure 1). The interaction between XIAP and the apoptotic form of AIF (Δ102) is highly dependant on the E3 ubiquitin ligase activity of XIAP. I determined that whereas wildtype XIAP has only weak affinity for Δ102 AIF, the XIAP variant H467A, which lacks the ability to ubiquitinate target molecules, is significantly enhanced in its ability to bind AIF (Figure 2). These data suggest that there is a transient, ubiquitin dependant association between XIAP and AIF, and that XIAP-mediated AIF ubiquitination may function to regulate the signaling properties of the AIF molecule. Further, I found that proteasomal inhibition did not result in an increase in endogenous AIF protein levels, and that XIAP-mediated ubiquitination of AIF did not result in AIF degradation (Figure 3). Taken together, these data suggest that the polyubiquitin chain formation that occurs on the AIF protein is mediated through lysine residues of ubiquitin other that K48, the predominant residue for targeting to the proteasome. In a separate line of investigation, I examined the ability of the XIAP antagonist Smac/DIABLO to displace AIF from interaction with XIAP, and determined that the overexpression of Smac/DIABLO prevented AIF from associating with XIAP (Figure 4). Examination of cellular lysates indicated that Smac/DIABLO overexpression induced an increase in the presence of high molecular weight AIF species, consistent with an increase in the amount of ubiquitinated AIF material. These data further support the hypothesis that the interaction between XIAP and AIF is highly dependant upon AIF ubiquitination status.
b) **Assessment of the effects of AIF on the caspase inhibitory properties of XIAP.** I determined that the caspase inhibitory properties of XIAP were unaffected by overexpression of either full-length, Δ54, or Δ102 AIF (Figure 5, see below for description of these AIF variants). This sub-aim was completed.
c) **Assessment of the effects of AIF on the ability of XIAP to augment TGF-β, JNK, or NF-κB signaling.** Experiments are ongoing, but were not completed during the project period.
d) **Evaluation of the effects of AIF on XIAP function in prostate tumor-derived cell lines.** Experiments are ongoing, but were not completed during the project period.
e) **Characterization of the effects of AIF on the copper metabolism regulatory properties of XIAP.** I found that the binding of copper by XIAP, which results in a profound conformational

change within the XIAP protein (Mufti et al, 2006), does not affect the ability of XIAP to bind AIF (Figure 6).

AIM 2. To determine the effects of XIAP on AIF function.

a) **Assessment of the effects of XIAP on the death promoting properties of AIF.** I determined that AIF overexpression does not induce apoptosis in multiple cell lines, including HeLa, Jurkat, and HEK293 (Figure 5). This sub-aim was completed.
b) **Determination of the caspase-dependence of the interaction between XIAP and AIF.** I determined that the caspase inhibitory properties of XIAP are dispensable for interaction with AIF, since the second BIR domain of XIAP (BIR2), which is incapable of inhibiting caspase activation, interacts with AIF to an extent comparable to that of wildtype XIAP or other BIR2-containing variants (Figure 7). This sub-aim was completed.
c) **Assessment of the effects of XIAP on the NADH-oxidase activity of AIF (Months 14-18).** I constructed plasmids to express recombinant AIF protein, in order to evaluate NADH-oxidase activity in the absence and presence of XIAP. Experiments are ongoing, but were not completed during the project period.
d) **Characterization of the ability of XIAP to ubiquitinate AIF.** I determined that XIAP is capable of ubiquitinating the full-length, Δ54, and Δ102 forms of AIF (Figure 8). Further observations are reported above for Aim1a. This sub-aim was completed.

In addition to the progress highlighted above, further discoveries were made that relate to the biological functions of XIAP and AIF, which are supplemental to the tasks outlined in my proposal. The amino-terminus of the mature form of AIF was found to begin at amino acid residue 55 (Δ54 AIF, Figure 9), resulting in a significantly larger protein than what was first reported during the original cloning of AIF (Susin et al., 1999), which suggested that the mature amino-terminus begins at amino acid residue 103 (Δ102 AIF). It has recently been reported that the release of AIF from the mitochondria during apoptosis requires two distinct events (Otera et al, 2005), consistent with my observations. The first is mitochondrial outer membrane permeablization (MOMP), a process that is regulated predominantly by members of the Bcl-2 family of proteins. The second is proteolytic cleavage by an as yet undefined protease, most likely of the serine protease family. In light of this report, I determined that the XIAP antagonist Omi/HtrA2, which possesses serine protease activity, is capable of cleaving the AIF protein in vitro (Figure 10). Given the ability of XIAP to interact with both Omi/HtrA2 and AIF, this raised the possibility that XIAP regulates the second step of AIF mitochondrial release, potentially through regulating the activity of Omi/HtrA2. To test this hypothesis, I investigated the release of AIF from the mitochondrial of mouse embryonic fibroblasts (MEFs) derived from mice deficient in Omi/HtrA2. When compared to control wildtype MEFs, no obvious differences were observed in the release of AIF from Omi/HtrA2 deficient cells (Figure 11). Interestingly, there was a significant reduction in the release cytochrome *c*.

Since the forms or AIF present in cells under normal and apoptotic conditions may interact differently with XIAP, I examined the cellular localization of both the Δ54 and Δ102 proteins, and determined that both are diffusely cytoplasmic (Figure 12).

While investigating the effects of AIF on the formation of reactive oxygen species (ROS) in cells under both normal and apoptotic conditions, I found that overexpression of AIF alone is sufficient to significantly increase the amount of ROS present in cells under normal conditions (Figure 13). Furthermore, upon treating cells with an apoptotic stimulus, there was a synergistic increase in ROS formation. Taken together these data suggest that increased AIF expression significantly increases steady state levels of cellular ROS, which is further augmented when cells receive an apoptotic stimulus.

My original proposal presented data suggesting that AIF protein levels were dramatically altered in tissues derived from XIAP-deficient mice. The reciprocal experiment was performed in which XIAP protein levels were examined in tissues from the Harlequin (Hq) mouse, a strain in which AIF proteins

levels are reduced by 80% or more in all tissues. XIAP protein levels were found to be significantly elevated in tissues derived from Hq animals when compared to wildtype controls (Figure 14).

Finally, given the high degree of similarity between members of the inhibitor of apoptosis (IAP) family, of which XIAP is the best described member, the data presented characterizing the significant interaction between XIAP and AIF raised the possibility that AIF may bind other IAP family members. I tested this possibility by examining the ability of AIF to bind the XIAP homologues cIAP-1 and cIAP-2, and determined that both IAP proteins interact strongly with AIF (Figure 15), suggesting that AIF may be a general purpose IAP binding protein.

Key Research Accomplishments

- XIAP was shown to preferentially interact with the Δ102 form of AIF in living cells
- It was shown that all forms of AIF (full-length, Δ54, and Δ102) fail to prevent XIAP-mediated caspase inhibition
- When overexpressed in a variety of cell lines, it was determined that all tested forms of AIF fail to induce cell death
- AIF was shown to be a substrate for XIAP-mediated ubiquitination
- The generally weak interaction between XIAP and the apoptotic (Δ102) form of AIF was found to be stabilized when the E3 ubiquitin ligase activity of XIAP was disrupted
- Steady state AIF protein levels were shown to be unaffected by proteasomal inhibition, and XIAP-mediated ubiquitination of AIF was shown not to result in AIF degradation
- The AIF/XIAP interaction was shown to be disrupted by the overexpression of Smac/DIABLO, likely due to increased AIF ubiquitination
- The amino-terminus of mature AIF in healthy cells was shown to begin at amino acid 55
- Truncation AIF variants (Δ54, Δ102) were shown to be cytoplasmically localized in living cells
- AIF overexpression was determined to increase basal ROS levels, and synergistically increase ROS formation following exposure of cells to etoposide
- XIAP protein levels were determined to be elevated in tissues from the Hq strain of mouse.
- Copper bound XIAP was determined to retain the ability to bind AIF
- AIF was shown to be a substrate for cleavage by the drosophila homologue of the IAP antagonist Omi/HtrA2, but no effects on the release of AIF from mitochondria were observed in Omi/HtrA2 deficient cells
- Delayed cytochrome *c* release was observed in cells deficient in Omi/HtrA2
- AIF was shown to robustly associate with the XIAP homologues cIAP-1 and cIAP-2

Reportable Outcomes/Bibliography

The following abstracts were presented at the 2005 Keystone Symposia on Cellular Senescence and Cell Death and the 2006 Gordon Conference on Cell Death, respectively:

- **Wilkinson, J.C.**, Wilkinson, A.S., Scott, F.L., Csomos, R.A., Salvesen, G.S. and Duckett, C.S. Neutralization of Smac/DIABLO by IAPs: a caspase-independent mechanism for apoptotic inhibition.
- **Wilkinson, J.C.**, Wilkinson, A.S., and Duckett, C.S. AIF is an XIAP interacting protein and target for XIAP-mediated ubiquitination.

While no peer-reviewed journal publications resulted during the project period, two manuscripts describing work completed during the course of this project are currently in preparation for submission during 2007.

The research supported by this project formed the scientific basis upon which I, as principle investigator, was able to search for and achieve an independent faculty position as Assistant Professor in the Department of Biochemistry at Wake Forest University School of Medicine, Winston-Salem, NC.

Training Accomplishments

As outlined in my original training proposal, this fellowship has allowed me to continue to hone my technical skills, which now span a wide range of techniques from the biophysical analysis of protein-protein interactions to whole animal physiology studies. Trough ongoing collaborations within the University of Michigan Comprehensive Cancer Center (UMCCC), I have presented data at monthly meetings of the UMCCC Prostate Cancer Specialized Project of Research Excellence (SPORE) program. Feedback from these meetings has been invaluable in further defining my research objectives, and through direct participation in the SPORE program, I have been able to gain tremendous amount first hand knowledge regarding the biology of prostate cancer. I have had the opportunity to attend two major scientific meetings, the 2005 Keystone Symposia on Cellular Senescence and Cell Death and the 2006 Gordon Conference on Cell Death, both of which greatly expanded my perspective on the role of cell death in tumor biology. Finally, I am fortunate to have had the opportunity to train with Colin S. Duckett, Ph.D., a leader in the field of cell death research and its application to cancer biology. He has passed on to me his proven skills in the areas of developing and supervising research, as well as in the direct training of postdoctoral fellows and graduate students. As highlighted above, this training has allowed me to recently obtain an independent faculty position. I continue to be devoted to the application of cell death research towards understanding the problem of prostate cancer, and I feel that based on my training supported by this fellowship I am prepared to undertake this task.

Conclusions

Based on the progress, current conclusions include: 1) XIAP and AIF interact in living cells under non-apoptotic conditions, 2) AIF does not alter the caspase inhibitory properties of XIAP, 3) the caspase inhibitory properties of XIAP are not required for interaction with AIF, 4) AIF is a substrate of XIAP-mediated ubiquitination, 5) the mature amino-terminus of AIF begins at amino acid 55, 6) the Δ54 and Δ102 truncations of AIF are cytoplasmically localized, 7) AIF overexpression results in increased ROS formation that is further amplified during apoptosis, and 8) XIAP protein levels are significantly altered in tissues from Hq (AIF deficient) mice, 9) the interaction between XIAP and AIF is dependant, in part, upon the ubiquitination status of AIF, 10) XIAP-mediated ubiquitination of AIF does not result in AIF degradation by the proteasome,11) the XIAP antagonist Smac/DIABLO interferes with the ability of AIF to bind XIAP possibly through increasing AIF ubiquitination, 12) the copper bound form of XIAP retains the ability to bind AIF, 13) AIF is a substrate for cleavage by the serine protease/XIAP antagonist Omi/HtrA2, but mitochondrial release of AIF does not appear to depend on Omi/HtrA2 activity, and 14) cytochrome *c* release is delayed in Omi/HtrA2 deficient cells, 15) AIF associates robustly with the XIAP homologues cIAP-1 and cIAP-2. Taken together, these observations support the relevance on the interaction between XIAP and AIF, and suggest that the properties of AIF span far beyond the induction of the apoptotic program. Thus AIF may have a profound affect on the process of tumorigenesis beyond simply regulating the cell death process.

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Otera H, Ohsakaya S, Nagaura Z, Ishihara N, & Mihara K. Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *EMBO J* **24(7)**, 1375-1386 (2005).

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. & Kroemer, G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446 (1999).

Appendix

All funds were used solely for the support (salary and fringe benefits) of the principle investigator, John C. Wilkinson, Ph.D. (100% effort)

Abstract presented at the 2005 Keystone Symposia on Cellular Senescence and Cell Death:

Neutralization of Smac/DIABLO by IAPs: a caspase-independent mechanism for apoptotic inhibition

John C. Wilkinson[1], Amanda S. Wilkinson[1], Fiona L. Scott[2], Rebecca A. Csomos[1], Guy S. Salvesen[2], Colin S. Duckett[1,3], Departments of Pathology[1] and Internal Medicine[3], University of Michigan, Ann Arbor, MI, 48109; The Program in Apoptosis and Cell Death Research[2], Burnham Institute, La Jolla, CA, 92037

Numerous members of the IAP family can suppress apoptotic cell death in physiological settings. While certain IAPs directly inhibit caspases, the chief proteolytic effectors of apoptosis, the protective effects of other IAPs do not correlate well with their caspase inhibitory activities, suggesting the involvement of alternative cytoprotective abilities. To examine this issue, we have characterized the protective effects of an ancestral, baculoviral IAP (Op-IAP) in mammalian cells. We show that although Op-IAP potently inhibited Bax-mediated apoptosis in human cells, Op-IAP failed to directly inhibit mammalian caspases. However, Op-IAP efficiently bound the IAP antagonist Smac/Diablo, thereby preventing Smac/Diablo-mediated inhibition of cellular IAPs. While reduction of Smac/Diablo protein levels in the absence of Op-IAP prevented Bax-mediated apoptosis, overexpression of Smac/Diablo neutralized Op-IAP-mediated protection, and Op-IAP variants unable to bind Smac/Diablo failed to prevent apoptosis. Finally, Op-IAP catalyzed the ubiquitination of Smac/Diablo, an activity that contributed to Op-IAP-mediated inhibition of apoptosis. These data show that cytoprotective IAPs can inhibit apoptosis through the neutralization of IAP antagonists, rather than by directly inhibiting caspases.

This work was supported in part by the University of Michigan Biomedical Scholars Program (to C.S.D.), grant T32 CA09676 (to J.C.W.) from the National Institutes of Health, grant W81XWH-04-1-0854 (to J.C.W.) from the Department of Defense Prostate Cancer Research Program, and a fellowship (to F.L.S.) from the National Health and Medical Research Council (Australia).

Abstract presented at the 2006 Gordon Conference on Cell Death:

AIF is an XIAP interacting protein and target for XIAP-mediated ubiquitination

John C. Wilkinson[1], Amanda S. Wilkinson[1], and Colin S. Duckett[1,2]. Departments of Pathology[1] and Internal Medicine[2], University of Michigan, Ann Arbor, MI, 48109

X-linked inhibitor of apoptosis (XIAP) is a well characterized inhibitor of cell death that protects cells by both caspase-dependent and independent mechanisms. In a biochemical screen for molecules that participate with XIAP in regulating cellular processes, we identified apoptosis inducing factor (AIF) as an XIAP binding protein. Domain analysis revealed that BIR2 of XIAP is both necessary and sufficient for interaction with AIF, and an XIAP variant incapable of binding caspases retained the ability to interact with AIF, suggesting that AIF employs a fundamentally different binding mechanism than other XIAP-associated factors. Human AIF localized to mitochondria, and the form present in healthy cells was determined to lack first 54 amino acids, differing significantly from the apoptotic form previously reported that lacks the first 102 amino-terminal residues. Fluorescence complementation and immunoprecipitation experiments revealed that XIAP is capable of interacting with both the healthy (delta 54) and apoptotic (delta 102) forms of AIF. Unlike other mitochondrial IAP antagonists, AIF failed to abrogate XIAP-mediated inhibition of caspase activity or prevention of apoptosis. Interestingly, AIF was found to be a target of XIAP-mediated ubiquitination, and an XIAP variant lacking E3 ubiquitin ligase activity displayed a more robust interaction with AIF. Expression of either XIAP or AIF reduced cellular ROS levels, and when expressed in combination, a synergistic decrease in both basal and peroxide-stimulated ROS was observed. Overall, these results identify AIF as a new XIAP binding partner, and suggest that in addition to its role in regulating caspase activation, XIAP regulates cellular ROS levels through interaction with AIF.

This work was supported in part by the University of Michigan Biomedical Scholars Program (to C.S.D.), and grant W81XWH-04-1-0854 (to J.C.W.) from the Department of Defense Prostate Cancer Research Program,

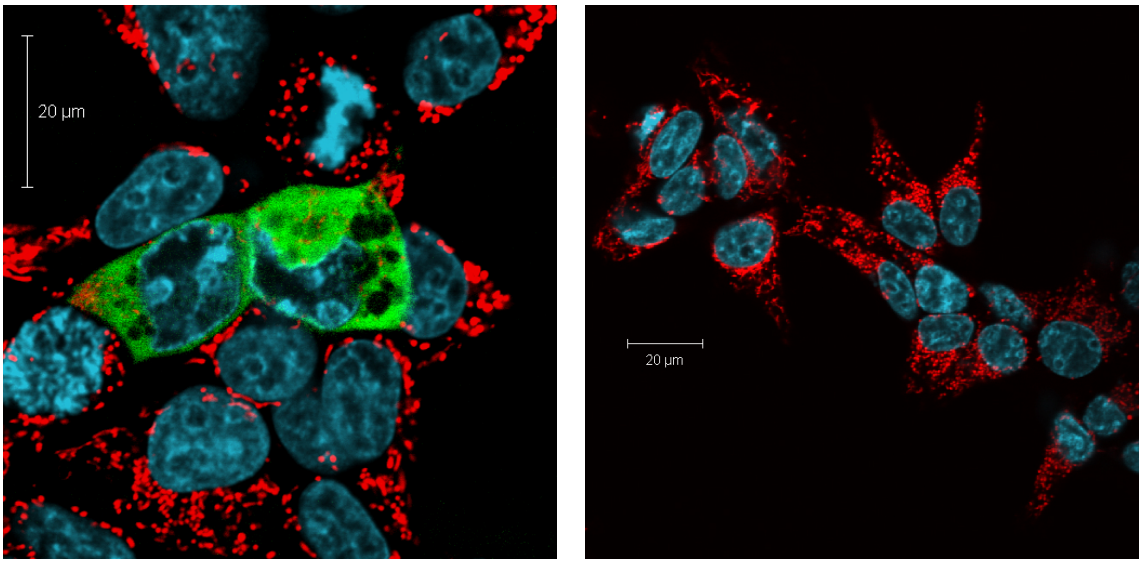


Figure 1. XIAP interacts preferentially with Δ102-AIF. HEK293 cells were transiently transfected with an expression plasmid encoding XIAP fused amino-terminally with the amino terminal domain of YFP (YN-XIAP) along with expression plasmids encoding either Δ102-AIF (left panel), Δ54-AIF (right panel), or full-length AIF (data not shown) fused carboxy-terminally with the carboxy terminal domain of YFP (Δ102-AIF-YC, Δ54-AIF-YC, FL-AIF-YC). In this system, only cells in which an interaction occurs between XIAP and AIF will emit YFP fluorescence (shown as green in these images). Cells were co-stained with both Mitotracker Red (mitochondrial marker, red fluorescence) and Hoescht (nuclear marker, blue fluorescence), and fluorescence was observed using a Zeiss LSM 510 confocal microscope. Note the significant levels of green fluorescence, indicative of XIAP/AIF binding in vivo, evident only in the XIAP/Δ102-AIF sample (left panel). Both D54-AIF-YC (right panel) and FL-AIF-YC (not shown) failed to produce fluorescence in combination with YN-XIAP. YN-XIAP/Δ102-AIF-YC signals were only detected in the cytoplasm, as confirmed by the absence of YFP signal localization with Mitotracker Red and Hoescht dyes.

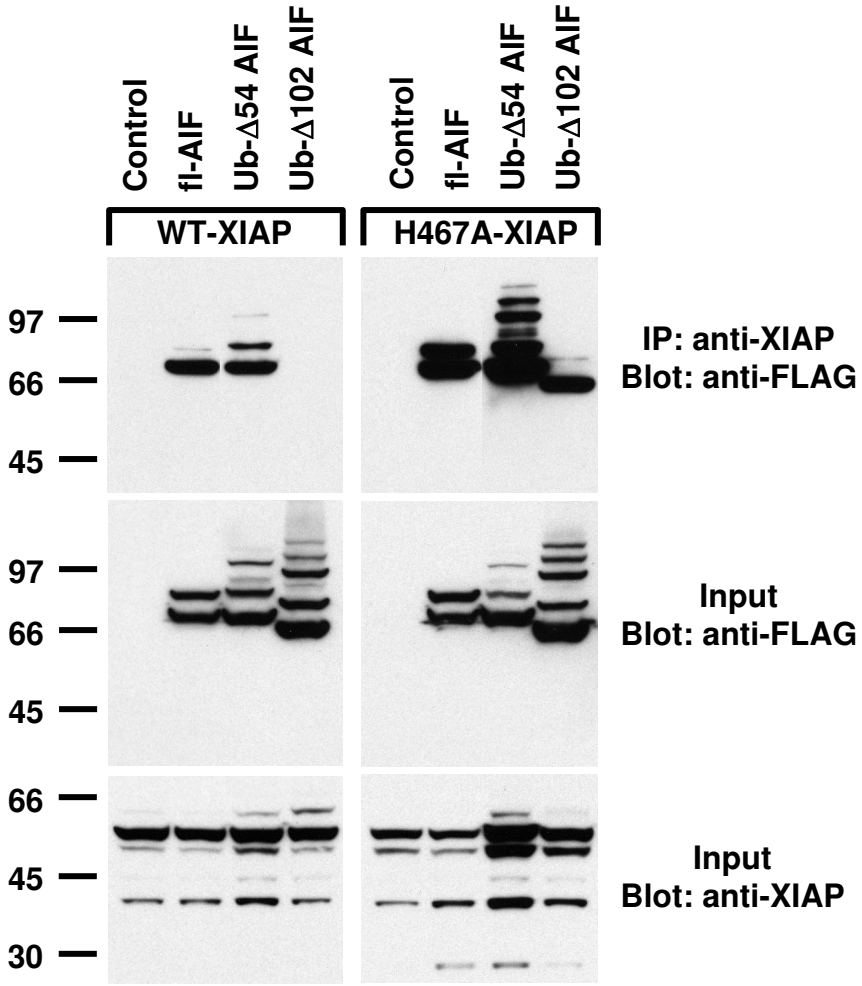
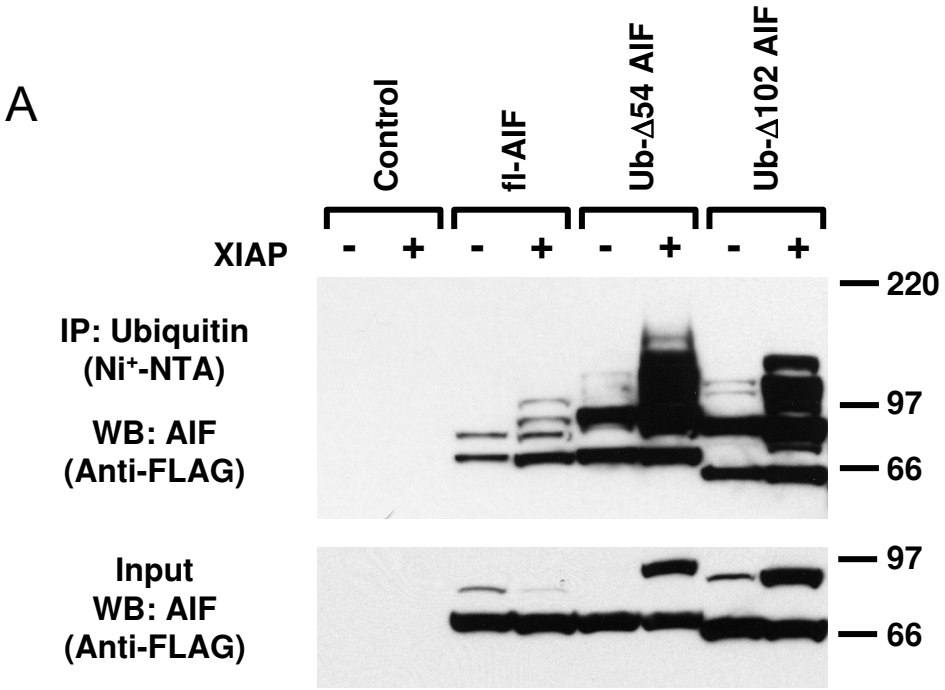


Figure 2: AIF association with wildtype, H467A XIAP. HEK 293 cells were transiently transfected with plasmids encoding the indicated proteins. Cellular lysates were prepared and XIAP was precipitated with an XIAP-specific antibody. The presence of AIF in precipitated complexes was determined by immunoblotting for the FLAG tag present at the carboxy terminus of each AIF protein. Note that both wildtype and the E3 ubiquitin ligase deficient (H467A) forms of XIAP robustly associate with full-length (fl) and Δ54 AIF, but only H467A-XIAP binds strongly to Δ102-AIF.



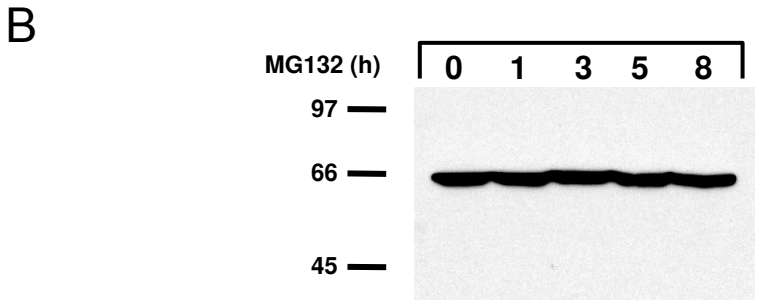


Figure 3: Ubiquitination of AIF does not result in degradation. Panel A: HEK 293 cells were transiently transfected with His-tagged ubiquitin and the indicated plasmids. Ubiquitinated material was then precipitated using Ni^{2+}-NTA beads, and the presence of FLAG-tagged proteins (AIF) in precipitated complexes (IP) was detected by immunoblot analysis (WB). Note that XIAP-mediated ubiquitination of AIF does not result in a decrease in recovered AIF protein (lower panel). Further note, this data was included in year one annual summary report, but is shown again here to highlight the lack of AIF degradation. Panel B: untransfected HEK 293 cells were treated with the proteasomal inhibitor MG132 for the indicated amounts of time. Cellular lysates were prepared and immunoblotted for the presence of AIF protein. Note the lack of AIF accumulation following MG132 treatment.

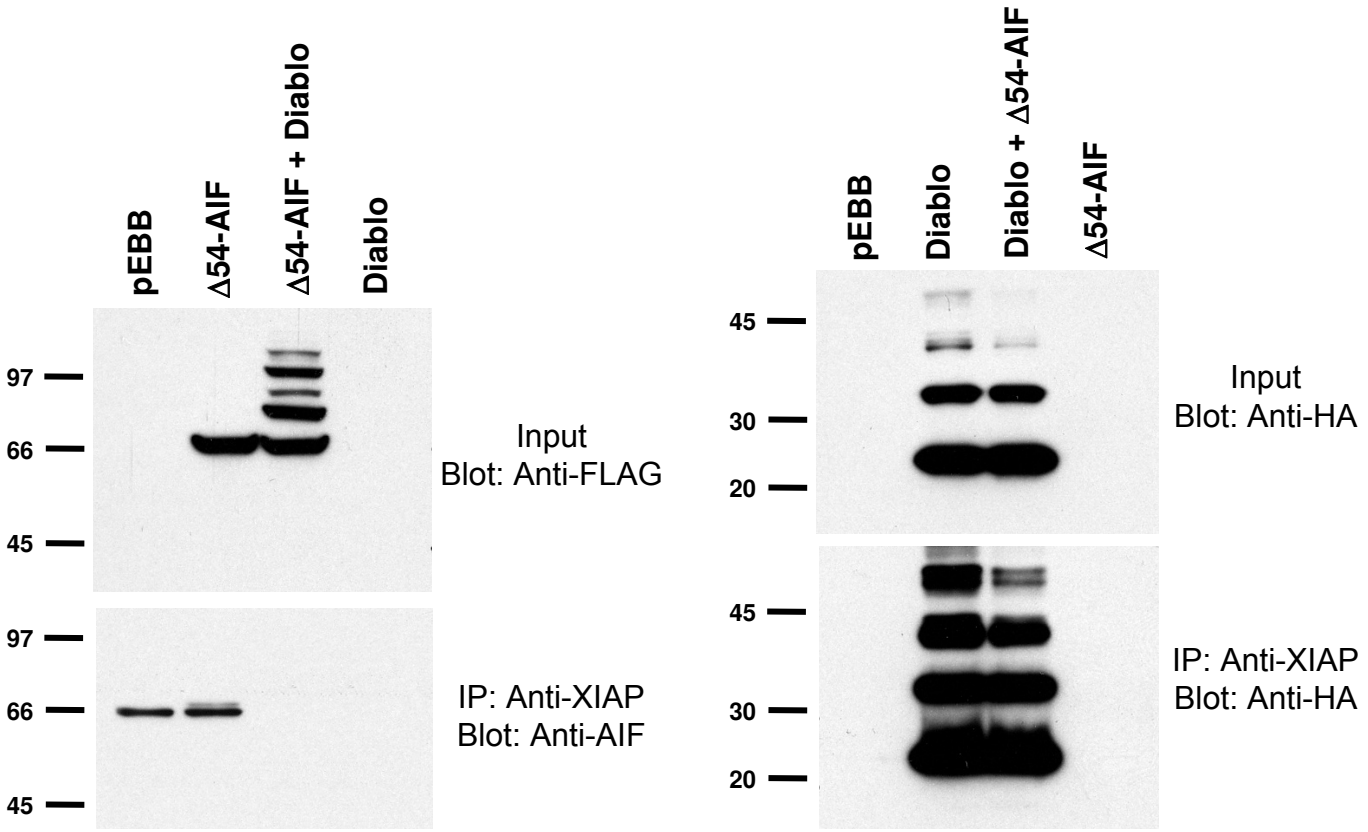


Figure 4: Smac/DIABLO disrupts XIAP-AIF association, increases AIF ubiquitination. HEK 293 cells were transiently transfected with a plasmid encoding XIAP in the absence and presence of Δ54-AIF-FLAG, Smac/DIABLO-HA, or both Δ54-AIF-FLAG and Smac/DIABLO-HA. Cell lysates were prepared and precipitated with an XIAP-specific antibody. Precipitated complexes were then immunoblotted for the presence of AIF (left panel) or Smac/DIABLO (right panel). Note the disruption of the association between XIAP and AIF as a result of Smac/DIABLO expression (left, lower panel), as well as the increase in AIF ubiquitination status following Smac/DIABLO co-expression (left, upper panel). The association between XIAP and Smac/DIABLO appears to be unaffected by the co-expression of AIF (right, upper panel).

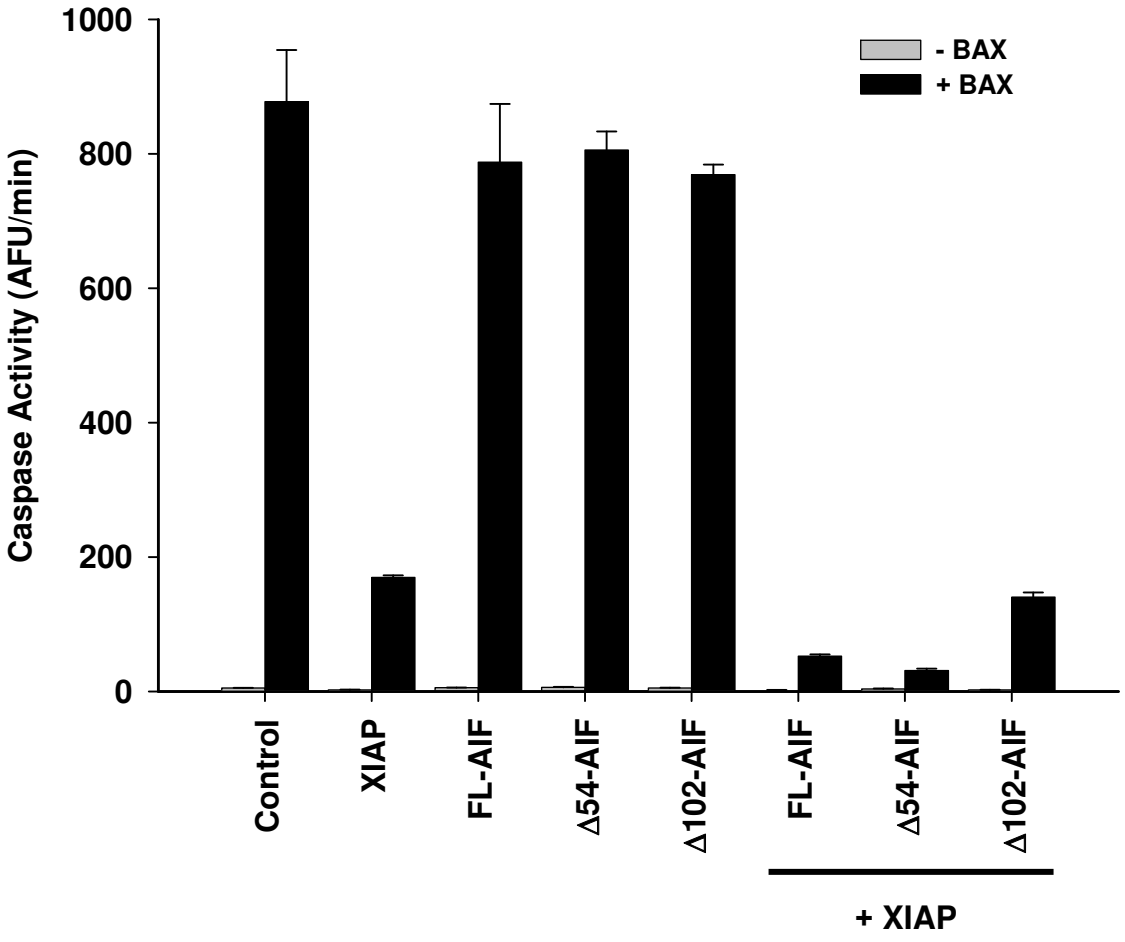


Figure 5. AIF does not abrogate XIAP-mediated caspase inhibition following Bax overexpression. HEK293 cells were transiently transfected with control, XIAP, or AIF (full-length AIF, ∆54-AIF, ∆102-AIF) expression plasmids in the absence (grey bars) or presence (black bars) of Bax. Additional samples in which AIF plasmids were co-transfected with XIAP were also prepared. Eighteen hours after transfection, cell lysates were prepared and tested for the presence of caspase-3 activity by incubation with the fluorogenic substrate DEVD-AFC. The averages +/- S.D. of multiple independent measurements are shown, and data are representative of at least three experiments.

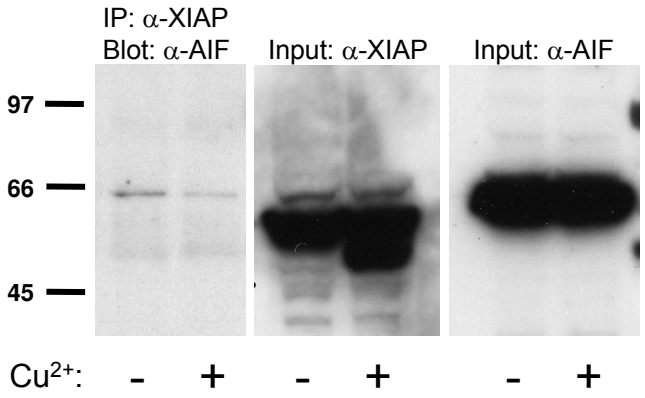


Figure 6: Copper shifted XIAP retains AIF binding capacity. HEK 293 were treated for 48 hours with copper sulfate in order to induce an electrophoretic mobility shift in XIAP (middle panel right lane), a phenomenon consistent with XIAP copper binding. Cells were then lysed, and XIAP was precipitated using an XIAP-specific antibody. The presence of AIF in precipitated material was then determined by immunoblotting. Note the precipitation of AIF (left panel) was unaffected by the copper-induced conformational change in XIAP. Also note that no change in AIF protein levels was observed following copper treatment (right panel).

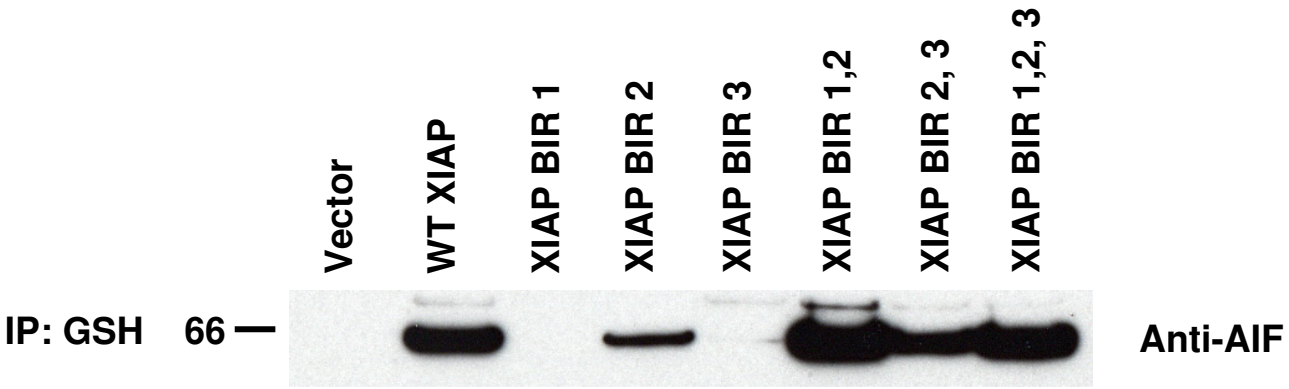


Figure 7. BIR2 of XIAP binds AIF. HEK 293 cells were transfected with plasmids encoding various XIAP domain truncations in fusion with GST along with a plasmid encoding full-length human AIF. Cell lysates were prepared, and XIAP was precipitated with glutathione beads. The presence of AIF in precipitated complexes was determined by immunoblotting.

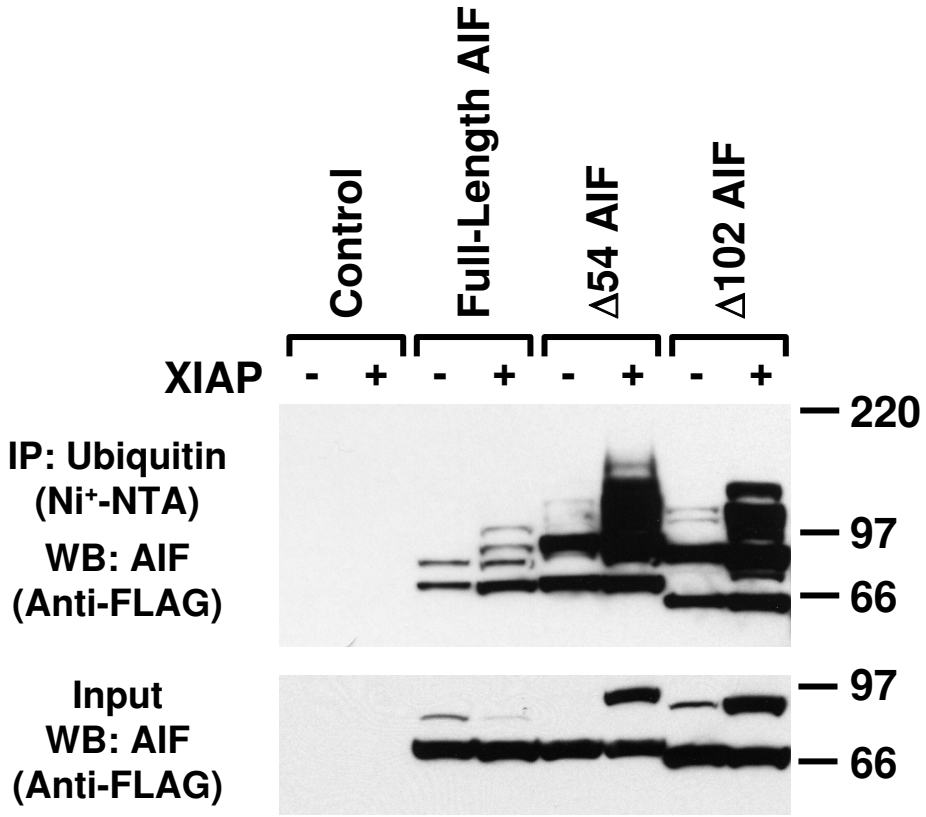
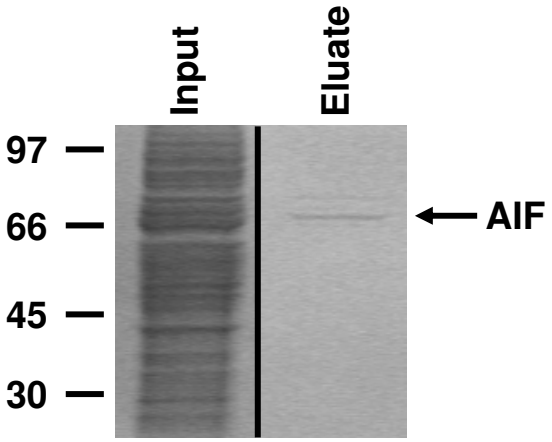


Figure 8. AIF is a substrate of XIAP-mediated ubiquitination. HEK293 cells were transiently transfected with His-tagged ubiquitin and either control of C-terminal FLAG-tagged AIF variants (full-length, Δ54-AIF, Δ102-AIF) in the absence and presence of XIAP. Ubiquitinated material was then precipitated using Ni^{2+}-NTA beads, and the presence of FLAG-tagged proteins (AIF) in precipitated complexes (IP) was detected by immunoblot analysis (WB). Equivalent protein expression for all AIF variants was confirmed by immunoblot analysis of input samples using anti-FLAG.

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  1 MFRCGGLAAG ALKQKLVPLV RTVCVRSPRQ RNRLPGNLFQ RWHVPLELQM TRQMASSGAS
 61 GGKIDNSVLV LIVGLSTVGA GAYAYKTMKE DEKRYNERIS GLGLTPEQKQ KKAALSASEG
121 EEVPQDKAPS HVPFLLIGGG TAAFAAARSI RARDPGARVL IVSEDPELPY MRPPLSKELW
181 FSDDPNVTKT LRFKQWNGKE RSIYFQPPSF YVSAQDLPHI ENGGVAVLTG KKVVQLDVRD
241 NMVKLNDGSQ ITYEKCLIAT GGTPRSLSAI DRAGAEVKSR TTLFRKIGDF RSLEKISREV
301 KSITIIGGGF LGSELACALG RKARALGTEV IQLFPEKGNM GKILPEYLSN WTMEKVRREG
361 VKVMPNAIVQ SVGVSSGKLL IKLKDGRKVE TDHIVAAVGL EPNVELAKTG GLEIDSDFGG
421 FRVNAELQAR SNIWVAGDAA CFYDIKLGRR RVEHHDHAVV SGRLAGENMT GAAKPYWHQS
481 MFWSDLGPDV GYEAIGLVDS SLPTVGVFAK ATAQDNPKSA TEQSGTGIRS ESETESEASE
541 ITIPPSTPAV PQAPVQGEDY GKGVIFYLRD KVVVGIVLWN IFNRMPIARK IIKDGEQHED
601 LNEVAKLFNI HED.
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Figure 9. The amino-terminus of mature AIF begins at amino acid 55. Top panel: HEK293 cells were transiently transfected with an expression plasmid encoding full-length AIF in fusion with a carboxy-terminal tandem affinity purification (TAP) tag. A cellular lysate was prepared (Input), and purified AIF-TAP (eluate) was subjected to Edmund degradation in order to determine the mature amino terminus. Bottom panel: the primary amino acid sequence of human AIF is displayed. The arrow indicates the cleavage site generating the mature amino-terminus, as determined from Edmund degredation of purified AIF-TAP.

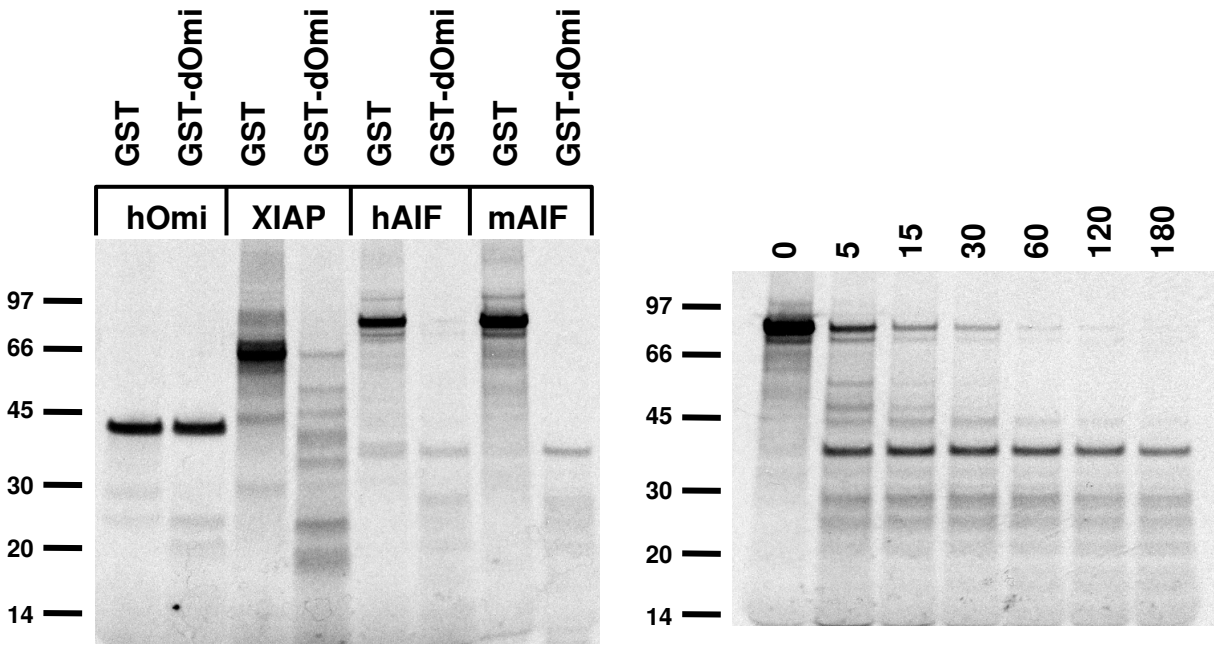

Figure 10: AIF is a substrate for Omi/Htra2 proteolysis. Left panel: ^{35}S-labeled, in vitro translated human Omi, XIAP, human AIF, and mouse AIF were incubated in the presence of recombinant glutathione-S-transferase (GST), or GST-tagged drosophila Omi for 4 h at 37 °C. The cleavage of substrate proteins was then determined following SDS-PAGE and autoradiography. Note that GST-Omi was able to induce cleavage in all substrates but Omi itself. Right panel: human AIF was incubated with GST-Omi at 37 °C for various amounts of time from 0 to 180 minutes. The cleavage of AIF was then determined by SDS-PAGE followed by autoradiography.

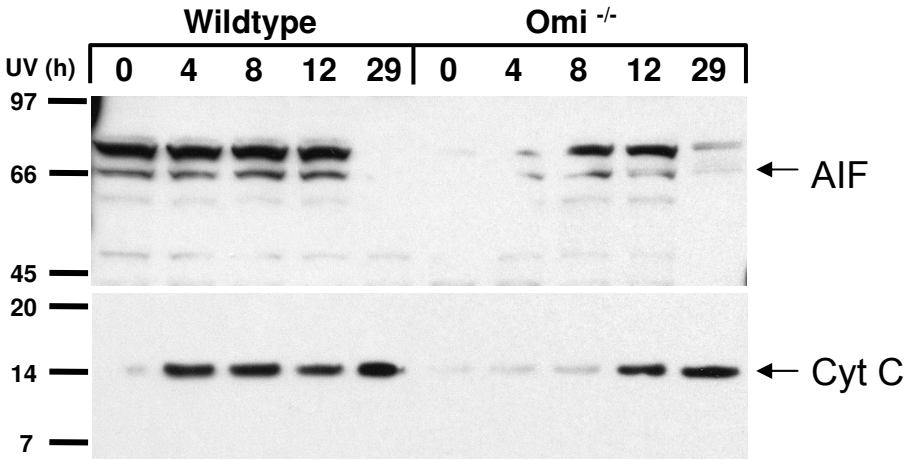

Figure 11: AIF release is unaffected, cytochrome *c* release is delayed in Omi knockout cells. Murine embryonic fibroblasts (MEFs) from wildtype or Omi knockout mice were treated with ultraviolet radiation. Cytoplasmic extracts were then prepared at various amounts of time following treatment, and the presence of both AIF (top panel) and cytochrome *c* (bottom panel) in the cytoplasm was determined by immunoblotting. Note that whereas the release of AIF appears unaffected (compare band intensity at 8 and 12 hours, ignoring transfer bubble present at 0 and 4 hours), the release of cytochrome *c* from Omi knockout cells is delayed when compared to wildtype controls.

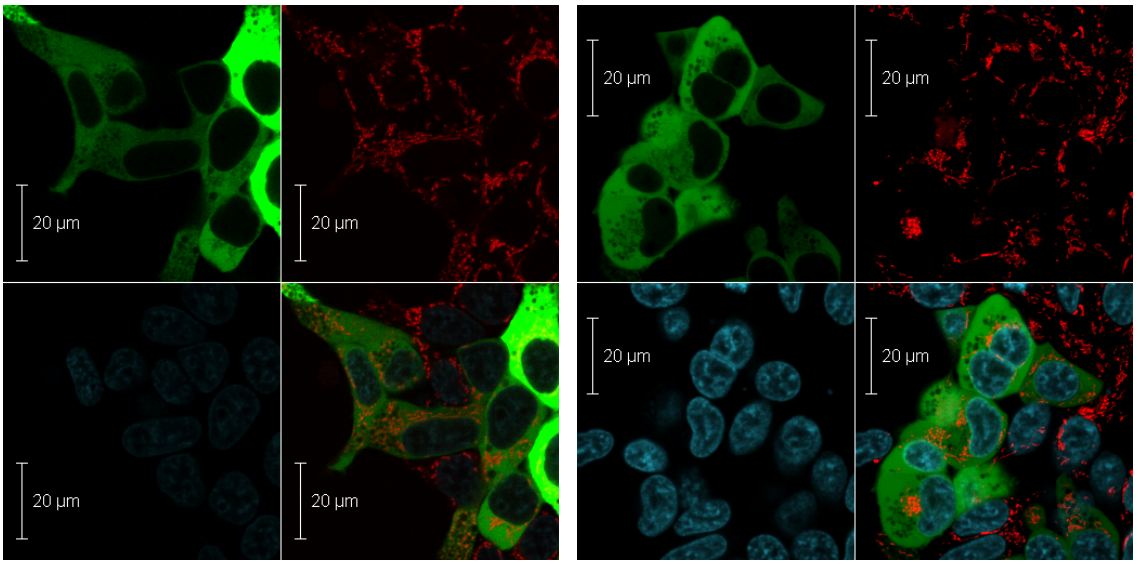

Figure 12. AIF truncation variants are cytoplasmically localized. HEK293 cells were transiently transfected with expression plasmids encoding either Δ54-AIF (left panel) or Δ102-AIF (right panel) in fusion with YFP (green fluorescence in these images) at the carboxy terminus. Cells were co-stained with both Mitotracker Red (mitochondrial marker, red fluorescence) and Hoescht (nuclear marker, blue fluorescence), and fluorescence was observed using a Zeiss LSM 510 confocal microscope. Note the cytoplasmic localization of both proteins, as evidenced by the lack of signal co-localization with either Mitotracker Red or Hoescht dyes.

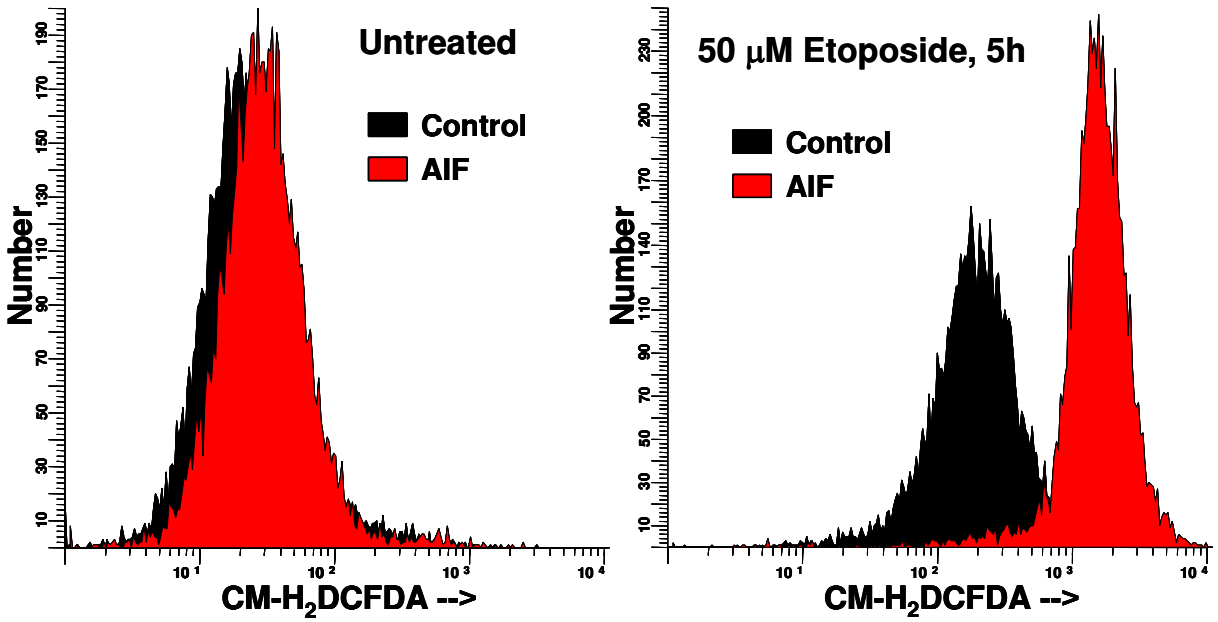

Figure 13. ROS formation in Jurkat cells overexpressing AIF. Jurkat cells were transiently transfected with either control (black) or AIF (red) expression plasmids. Forty eight hours after transfection, cells were left untreated (left panel) or treated with etoposide (right panel) for 5 h. Following treatment, cells were harvested and stained with the ROS indicator CM-H_2DCFDA, and ROS levels were quantified by flow cytometry.

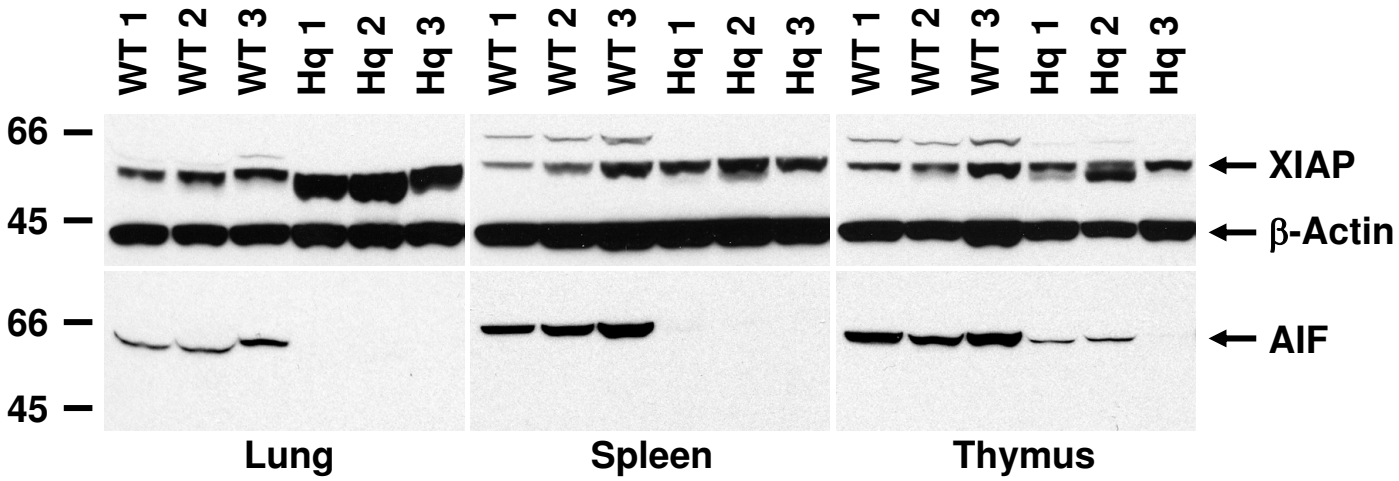

Figure 14. XIAP protein expression is tissues derived from Hq mice. Lung, spleen, and thymus were isolated from three Harlequin (Hq) and three corresponding wildtype (WT) littermate control mice. Protein extracts were prepared and immunoblotted for the presence of XIAP (top panel) and AIF (bottom panel). As a loading control, the top panel was also immunoblotted for the presence of β-actin. Note either the absence or reduction of AIF protein in all Hq tissues, as well as the increase in XIAP protein in Hq tissues, especially lung.

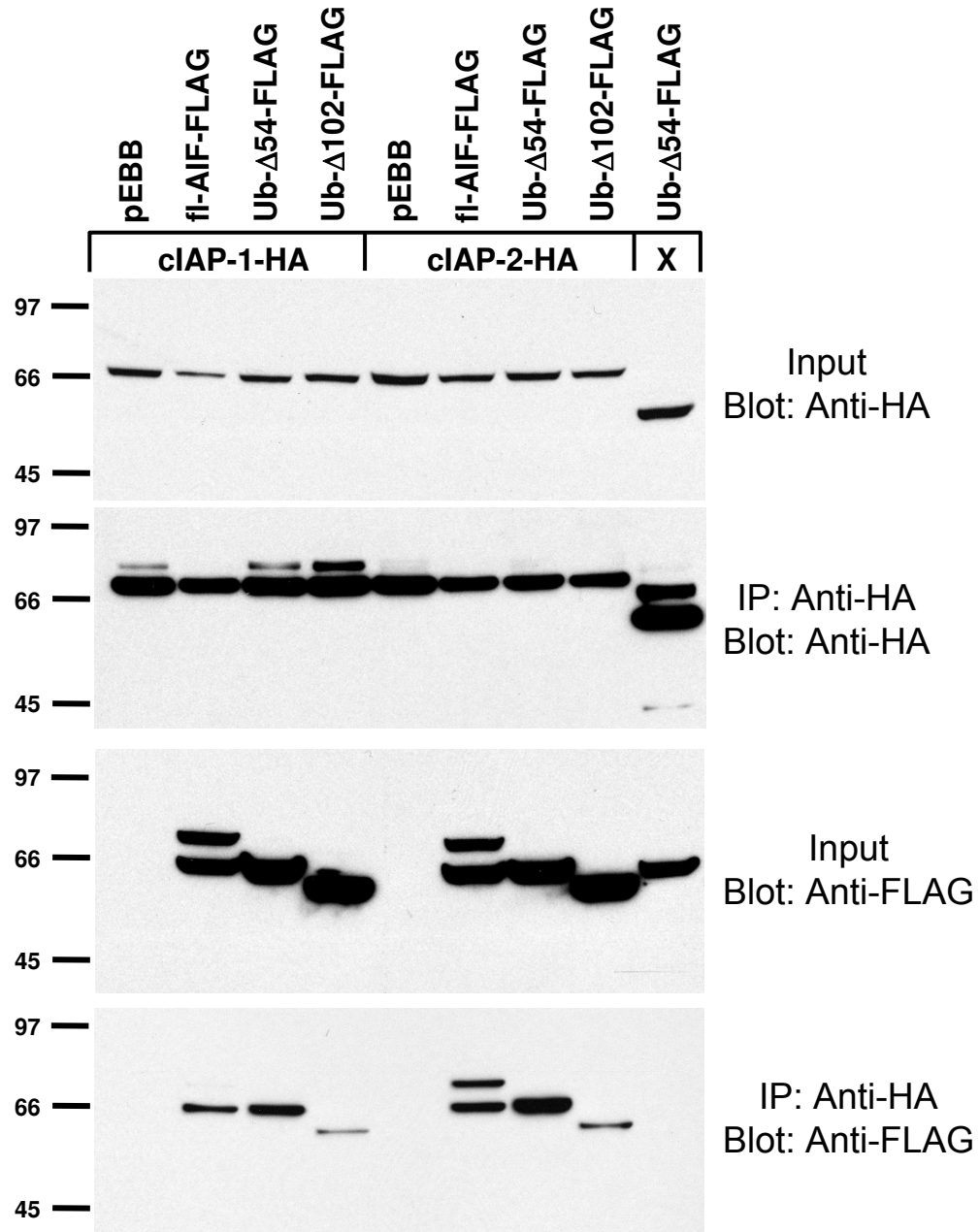

Figure 15: AIF binds the XIAP homologues cIAP-1 and cIAP-2. HEK 293 cells were transiently transfected with plasmids encoding HA-tagged cIAP-1 or cIAP-2 along with plasmids encoding full-length AIF-FLAG, Δ54-AIF-FLAG, or Δ102-AIF-FLAG. As a control, an additional sample was included in which cells were transfected with HA-tagged XIAP along with Δ54-AIF-FLAG. Cell lysates were prepared and IAP proteins were precipitated with anti-HA antibodies. The presence of AIF variants in immune complexes was determined by immunoblotting with a FLAG-specific antibody (bottom panel). Equivalent expression of all transfected proteins was determined by HA (top panel) and FLAG (lower middle panel) specific immunoblotting of input lysates, and precipitation of IAP proteins was confirmed by immunoblotting recovered material with an HA-specific antibody (upper middle panel). Note that all three AIF variants were efficiently precipitated by both cIAP-1 and cIAP-2, to an extent that was greater than that for XIAP.